JOURNAL OF VIROLOGY, Jan. 2009, p. 1018–1025 Vol. 83, No. 2
0022-538X/09/$08.00+0 doi:10.1128/JVI.01882-08

Functional Consequences of Human Immunodeficiency Virus Escape from an HLA-B*13-Restricted CD8$^+$ T-Cell Epitope in p1 Gag Protein$^\nabla$

Julia G. Prado,[1] Isobella Honeyborne,[1] Ian Brierley,[2] Maria Carmen Puertas,[3] Javier Martinez-Picado,[3,4] and Philip J. R. Goulder[1,5,6]*

Department of Paediatrics, University of Oxford, Peter Medawar Building for Pathogen Research, South Parks Rd., Oxford OX1 3SY, United Kingdom[1]; Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QP, United Kingdom[2]; Fundació IrsiCaixa, Hospital Universitari Germans Trias i Pujol, Badalona, Spain[3]; Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain[4]; HIV Pathogenesis Programme, Doris Duke Medical Research Institute, University of KwaZulu-Natal, Durban 4013, South Africa[5]; and Partners AIDS Research Center, Massachusetts General Hospital, 13th St., Bldg. 149, Charlestown, Boston, Massachusetts 02129[6]

Received 8 September 2008/Accepted 13 October 2008

The observed association between HLA-B*13 and control of human immunodeficiency virus type 1 (HIV-1) infection has been linked to the number of Gag-specific HLA-B*13-restricted cytotoxic T-cell (CTL) responses identified. To date, the Gag escape mutations described that result in an in vitro fitness cost to the virus have been located within structural protein p24 only. Here we investigated the hypothesis that CTL escape mutations within other regions of HIV Gag may also reduce viral fitness and contribute to immune control. We analyzed an HLA-B*13-restricted CTL response toward an epitope in p1 Gag, RQANFLGKI$_{429\text{-}437}$ (RI9), where amino acid variation at Gag residues 436 and 437 is associated with HLA-B*13 expression. In this work, we assessed the impact of amino acid substitutions at these positions on CTL recognition and on HIV-1 fitness. We demonstrated that substitutions I437L and I437M largely abrogate CTL recognition and reduce viral fitness while variants K436R and I437V have only a marginal effect on recognition and fitness. Examination of the patterns of protein synthesis indicated that the loss of fitness in the I437L and I437M mutants is associated with the accumulation of unprocessed Gag precursors. A significant reduction in ribosomal frameshifting efficiency was observed with I437M, suggesting that this mechanism contributes to the observed reduced fitness of this virus. These studies illustrate the apparent trade-off available to the virus between evasion of CTL recognition in p1 Gag and the functional consequences for viral fitness.

CD8$^+$ cytotoxic T lymphocytes (CTLs) play a crucial role in controlling many viral infections (6). During the last decade, several studies have shown the importance of CTLs in controlling human immunodeficiency virus (HIV) and simian immunodeficiency virus replication (19, 36), including those describing the association of immune control with the expression of certain major histocompatibility complex (MHC) class I molecules. Most notably, these are HLA-B*57, -B*5801, -B*27, and -B*51 (19, 25, 33) in humans and Mamu-A*01, Mamu-B*17, and Mamu-B*08 (29, 37, 49) in macaques. Nevertheless, the precise characteristics of the CTL responses that confer this protective profile on particular MHC class I alleles remain unclear.

Gag-specific CTL responses have been strongly associated with low viremia in HIV infection (12, 22, 26, 51). One hypothesized mechanism is that Gag is highly immunogenic and yet relatively conserved, so that escape from Gag-specific responses may reduce viral replicative capacity. Thus, in spite of the extraordinary flexibility of HIV to escape from host immune pressure (3, 10, 41), Gag-specific escape may be of benefit to the host because of the cost of Gag escape to the virus.

Recent data have supported this hypothesis in demonstrating a link between the selection of Gag escape mutations that reduce in vitro viral fitness and the association of the selecting HLA class I alleles with long-term control of HIV (32). The HLA allele most consistently associated with effective control of HIV is HLA-B*57 (19). In vivo reversion of escape mutations after transmission to MHC-mismatched recipients has been described in three Gag B*57 epitopes, ISPRTLNAW (Gag$_{147\text{-}155}$, ISW9) (8), TSTLQEQIAW (Gag$_{240\text{-}249}$, TW10) (28), and KAFSPEVIPMF (Gag$_{162\text{-}172}$, KF11) (9), suggesting that these mutations in Gag are associated with a fitness cost. In vivo data have also been strongly supported by in vitro results (4, 9, 30). Moreover, the complexity of the mutational pathways required for HIV to overcome the fitness constraint of the Gag B*27-restricted KRWIILGLNK (Gag$_{263\text{-}272}$, KK10) response that is associated with successful long-term control of HIV (13, 18) has been revealed recently as one of the causes for late viral escape in B*27-positive individuals (45). The escape mutations within the ISW9, TW10, KF11, and KK10 CTL epitopes are all located within the HIV p24 capsid protein.

In this work, we focused on HLA-B*13, an allele previously shown to be associated with HIV-1 control (20, 22). One of the immunodominant HLA-B*13-restricted CTL epitopes, RQANFLGKI (Gag$_{429\text{-}437}$, RI9), targeted during chronic in-

* Corresponding author. Mailing address: Department of Paediatrics, University of Oxford, Peter Medawar Building for Pathogen Research, South Parks Rd., Oxford OX1 3SY, United Kingdom. Phone: 44 1865 281884. Fax: 44 1865 281236. E-mail: philip.goulder@paediatrics.ox.ac.uk.

$^\nabla$ Published ahead of print on 22 October 2008.

fection is located within the Gag protein, p1, a 16-amino-acid peptide to which no specific function has been attributed as yet. However, the p1 sequence forms part of the viral ribosomal frameshifting signal involved in Gag-Pol expression and the control of the Gag/Gag-Pol ratio, the maintenance of which is crucial to particle assembly, replication, and viral infectivity (24, 46). Furthermore, the p1 sequence contains a protease cleavage site at the nucleocapsid p7 (NC-p7)/p1 junction (40).

Previous studies have shown that amino acid variation at Gag residues 436 and 437 (that is, at positions 8 and 9 in the RI9 epitope) is strongly associated with HLA-B*13 ($P = 0.002$ and 2.41×10^{-7}, respectively) (22). In this study, we addressed the hypothesis that this amino acid variation within the HLA-B*13 RI9 epitope is selected to reduce CTL recognition but that the escape mutations also reduce viral replicative capacity. Further, we investigated the possibility that these variants may interfere with ribosomal frameshifting efficiency.

MATERIALS AND METHODS

Recognition of Gag RI9 variants. Peptides representing amino acid changes I437V/M/L and K436R and double mutations K436R/I437L and K436R/I437M were synthesized. Recognition of the variant peptides compared to wild-type RI9 was determined by measuring the gamma interferon (IFN-γ) response to serially diluted peptides for each variant in an enzyme-linked immunospot assay of peripheral blood mononuclear cells (PBMCs) obtained as previously described from an HLA-B*13-positive individual, 93-08024-RI, with wild-type responses to RI9 (17).

Construction of 2113 M-WT recombinant plasmid. We constructed a recombinant plasmid carrying the fragment of interest from a clade C wild-type virus-infected, HLA-B*13-positive subject (2113 M) since mutations in RI9 were identified in the context of clade C virus infection. Proviral DNA was extracted from PBMCs isolated from study subject 2113 M, and an 837-bp fragment was PCR amplified with primers ApaI (5′ACA TAG CCA GAA ATT GCA GGG CCC CTA G 3′; positions 1199 to 1226 of HIV-1_{HXB2}) and Sse8387 (5′ CTG ATT TTT TTC TGT TTT AAC CCT GCG GAT G 3′; positions 2865 to 2835 of HIV-1_{HXB2}). This PCR fragment was cloned into the pJM11ΔPR vector as previously described (31).

Site-directed mutagenesis. Mutations K436R, I437V, I437M, and I437L in RI9 were introduced by site-directed mutagenesis (QuikChange I; Stratagene, United Kingdom) into 2113 M recombinant plasmid already containing the wild-type sequence (2113 M-WT). The whole plasmid DNA was PCR amplified in a mutagenesis reaction with two overlapping primers containing the target mutation. The primers used for the mutagenesis reaction were I437V-F (5′-GG CAGGCTAATTTTTTAGGGAAAGTTTGGCCTTCCCA-3′ [the mutagenesis site is underlined]), I437V-R (5′-CCGTCCGATTAAAAAATCCCTTTCTTTG GCCTTCCCT-3′); I437L-F (5′-GGCAGGCTAATTTTTTAGGGAAACTTTG GCCTTCCCA-3′), I437L-R (5′-CCGTCCGATTAAAAAATCCCTTTGAAAC CGGAAGGGT-3′); I437M-F (5′-GGCAGGCTAATTTTTTAGGGAAAATG TGGCCTTCCCACA-3′), I437M-R (5′-CCGTCCGATTAAAAAATCCCTTT TACACCGGAAGGGTGT-3′); K436R-F (5′-GCAGGCTAATTTTTTAGGG AGAATTTGGCCTTCCCACAA-3′), and K436R-R (5′-CGTCCGATTAAAA AATCCCTCTTAAACCGGAAGGGTGTT-3′). The presence of mutations was verified by DNA sequencing of nucleotide positions 1,000 to 2,900 (HIV-1_{HXB2} reference) in newly generated plasmid clones. The DNA fragment ranging from the ApaI to the Sse restriction site was then subcloned into a new pJM11ΔPR vector to avoid potential carryover of additional mutations during mutagenesis, and the coding region sequence was verified again.

Virus production. Viral stocks were produced by cotransfection of the different 2113 M recombinant plasmids generated (5′ half of the HIV-1_{NL43} strain) with p83-10_{eGFP} (3′ half of the HIV-1_{NL43} strain) (48) in MT4 cells. Viral stocks were harvested, and viral RNA was extracted (Qiagen, United Kingdom). The *gag* and *pol* coding regions were PCR amplified and sequenced to confirm the presence of the introduced mutations and the absence of other potential variations. The 50% tissue culture infective dose of each viral stock was determined with MT4 cells by the Reed and Muench method (35).

Replication kinetics. Jurkat T cells were infected in triplicate at a multiplicity of infection (MOI) of 0.001 in a total volume of 3 ml with the wild-type virus or variants and incubated at 37°C for 2 h. Pellets were washed twice with phosphate-buffered saline and cultured at 37°C and 5% CO_2, as previously described (43). After infection, 150 μl of supernatant and at least 50,000 cells were harvested daily in order to measure p24 production by enzyme-linked immunosorbent assay (ELISA; Innogenetics, United Kingdom) and infectivity by determining the percentage of enhanced green fluorescent protein (eGFP)-positive cells by fluorescence-activated cell sorter (FACS) analysis. The growth rate and infectivity rate of each virus were analyzed by fitting a linear model to the $\log_{10}$-transformed data of p24 production and eGFP expression and comparing the slopes. The slope quantifies the steepness of the line for each linear regression. This is calculated by $\Delta Y/\Delta X$, where ΔY is the change in Y (p24 or eGFP increase) for each unit change in X (days after infection).

Western blot analysis. Jurkat T cells were infected at an MOI of 0.001 with the wild-type virus or variants and incubated at 37°C for 2 h. At days 1, 2, 3, 4, and 5, 1 ml of supernatant and cell pellet was sampled. Cell pellets for days 1, 2, 3, 4, and 5 were lysed, and 8 μg of total protein was loaded onto a sodium dodecyl sulfate (SDS)-polyacrylamide gel. In addition, virus particles were collected and purified from 500 μl of cell culture supernatant by pelleting through a 20% sucrose cushion (20,000 × *g*, 2 h, 4°C). Proteins from cell lysates and virions were transferred onto a nitrocellulose membrane (Invitrogen); probed with a mouse monoclonal p24 antibody (Abcam) and mouse monoclonal β-actin antibody (Chemicon), followed by incubation with anti-mouse immunoglobulin G peroxidase-linked whole antibody (Pierce); and developed with SuperSignal West femto (Pierce). Antigen-antibody complexes were detected by chemiluminescence, and bands were quantified with Molecular Imaging Software (Kodak).

Ribosomal frameshifting assays. A short (74-bp) DNA fragment encompassing the ribosomal frameshifting signal of 2113 M-WT (or a mutant variant) was amplified by PCR with IB15 (5′-CAGGTCGACAAGGCAGGCTAATTTTTT AGGG-3′ and IB16 (5′-CAGGGATCCTCTGGTCTGCTCTGAAGGAAA-3′) and cloned between the *Renilla* and firefly luciferase genes of dual-luciferase reporter plasmid p2luc (27) by using the SalI and BamHI restriction sites. The p2luc plasmid allows ribosomal frameshifting to be assayed in vitro in the rabbit reticulocyte lysate (RRL) in vitro translation system or in transfected tissue culture cells. For RRL translations, mRNAs were prepared from EcoRI-digested p2luc derivatives, and translation products were analyzed on SDS-10% (wt/vol) polyacrylamide gels as described previously (15). The relative abundance of nonframeshifted and frameshifted products on the gels was determined by direct measurement of [^{35}S]methionine incorporation with a Packard Instant Imager 2024 and adjusted to take into account the differential methionine contents of the products. In-culture frameshift assays used Cos-7 cells. The p2luc-2113 M series of plasmids were transfected by a commercial liposome method (FuGene 6; Roche). The cells were harvested 24 h posttransfection, and reporter gene expression was determined with a dual-luciferase assay system kit (Promega). Each data point represents the mean value (± the standard error of mean) of six separate transfections.

Statistical analysis. All statistical analysis was performed with Prism 4.0 (GraphPad).

RESULTS

HLA-B*13 CD8 T-cell response drives HIV-1 escape in Gag RI9. The variants selected preferentially in HLA-B*13-positive subjects within the epitope RQANFLGKI (Gag 429 to 437) arise at Ile-437 (in 30% of B*13 positives versus 3% of B*13 negatives) and at Lys-436 (21% of B*13 positives versus 7% of B*13 negatives) (22). In order to evaluate the effect on epitope recognition of mutations away from consensus for the common variants associated with B*13 expression, synthetic peptides representing amino acid changes I437V/M/L and K436R and double mutations K436R/I437L and K436R/I437M were synthesized. The IFN-γ response to serial dilutions of these peptides was measured by enzyme-linked immunospot assay with PBMCs (Fig. 1). A half-log increase in the peptide concentration required to induce half the maximal response was observed for variants with single amino acid substitutions at positions I437L and I437M (149 and 233 nM, respectively) compared with the wild-type RI9 peptide (30 nM). There was no evidence from these assays that the I437V variant (half-log

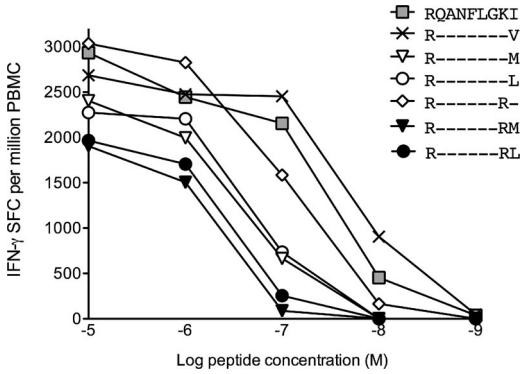

FIG. 1. IFN-γ responses to a panel of synthetic peptides containing RI9 variants. Shown is the IFN-γ response to the wild-type epitope RQANFLGKI (RI9) compared to the response to variants seen in HLA-B*13-positive individuals. Synthetic peptides were serially diluted, and the IFN-γ response made by PBMCs from individual 93-08024-RI (known to respond to the wild-type epitope) was measured in duplicate.

increase in the peptide concentration required to induce half the maximal response, 24 nM) was significantly less well recognized than the wild type, although it is notable that no HLA-B*13-positive individuals whose autologous virus encoded amino acid variation at residue 437 showed a response to RI9 in a previously described cohort study (22), suggesting the possibility that the I437V variant may impair recognition. Double-mutant variants K436R/I437L and K436R/I437M more seriously affected epitope recognition, having a 1-log decrease in peptide avidity. Thus, the amino acid changes at Gag residues 436 and 437 represent escape variants in the context of HLA-B*13-restricted RI9 responses.

Viral escape in B*13-RI9 affects in vitro HIV fitness. Since previous studies have shown the potential benefit of Gag-specific CTL responses that impose structural constraints on HIV by driving the selection of particular escape mutations (9, 28, 30), we investigated the impact of the RI9 escape mutations identified here on HIV-1 replication. Mutations K436R, I437V, I437M, and I437L in RI9 were introduced by site-directed mutagenesis into 2113 M-WT, a recombinant plasmid constructed from the viral DNA of an HLA-B*13-positive, HIV-infected study subject, 2113M. Viral stocks were produced by cotransfection of HIV-relevant plasmids into MT4 cells, and replication kinetics were examined in Jurkat cells at an MOI of 0.001. Following infection of cells, viral growth was monitored by p24 production (ELISA) and infectivity was measured as the percentage of eGFP-positive cells (FACS). A 1-log reduction in p24 production was observed for the I437M and I437L mutants compared with the wild type (Fig. 2a). In contrast, only minor differences in p24 production were observed for the I437V and K436R mutants. The same pattern was seen in the infectivity assay, where the I437M and I437L mutants showed reduced infectivity compared with the wild type and the I437V and K436R mutants (Fig. 2b).

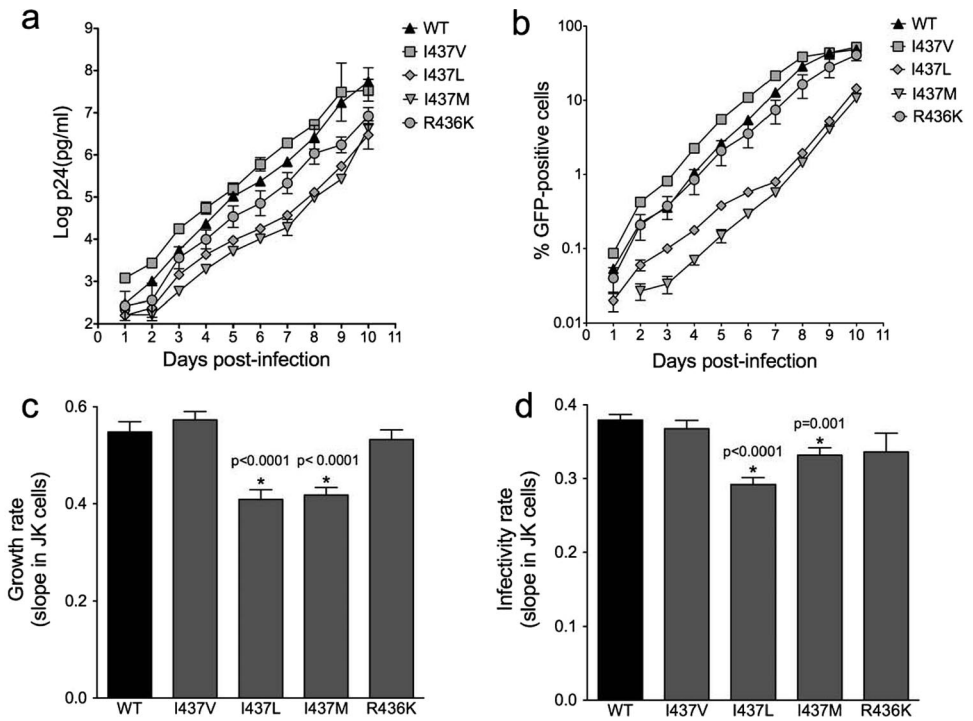

FIG. 2. Replication kinetics of HIV-1 RI9 escape mutants. Jurkat cells were infected in triplicate at an MOI of 0.001 with the I437V, I437L, I437M, and K436R viruses and compared to those infected with the wild type (WT) virus (2113 M). The replication kinetics of RI9 virus were followed daily after infection. (a) Viral growth was determined by p24 ELISA after infection. (b) Viral infectivity was measured by the percentage of eGFP$^+$ cells by FACS analysis. (c) The bar graph represents the growth rate calculated as the slope of log p24 measured by ELISA in the kinetics experiments. For details of the slope calculation, see Materials and Methods. (d) The infectivity rate was calculated as the slope of log eGFP$^+$ cells measured in the kinetics experiments. *P* values reflect comparisons of the variants to the wild type (values with asterisks indicate that the *P* values are still significant after Bonferroni correction).

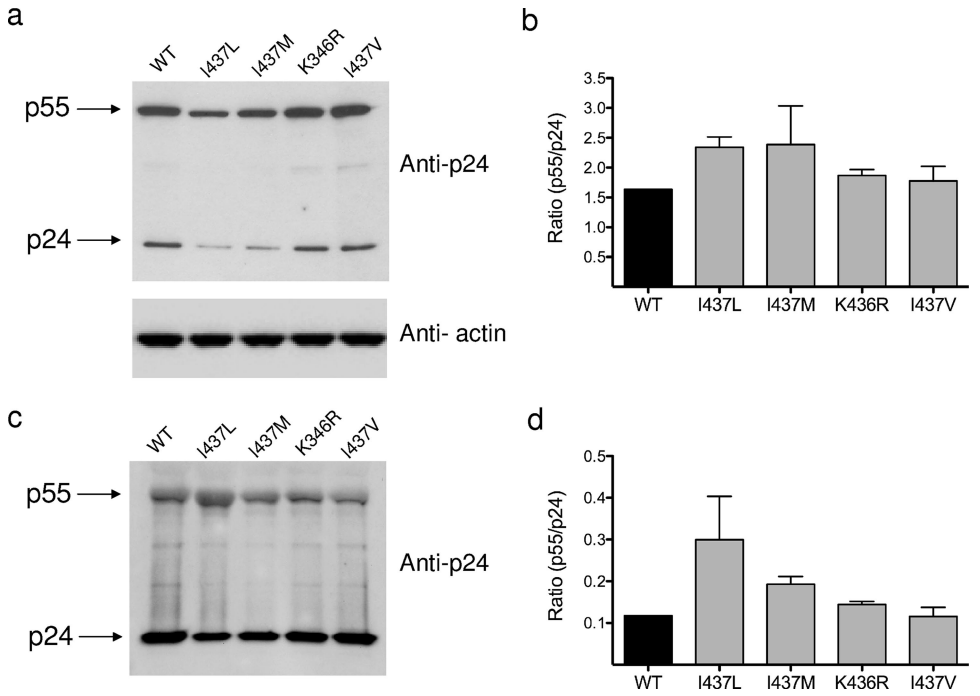

FIG. 3. Impact of RI9 mutations on HIV Gag expression as determined by Western blot assay of cells and virions. (a) Western blot assay of cell lysates at day 5 (representative of four independent blots). Total protein was extracted from cell pellets, subjected to SDS-PAGE, blotted onto nitrocellulose filters, and probed with anti-p24 and anti-actin antibodies. Bands corresponding to mature p24 protein and the unprocessed p55 Gag precursor are indicated by arrows. Anti-actin antibody acts as a loading control. (c) Western blot assay of supernatant virus. Details are as in panel a. (b and d) p55/p24 viral protein ratios quantified in cells (b) and virions (d). Graphs represent the mean quantification of four independent blots, and error bars represent the standard error of the mean. WT, wild type.

The growth and infectivity rates of the viral variants were calculated based on p24 production and eGFP expression in the exponential phase by fitting the log-transformed data into a linear model and comparing the slopes (50). There was a significant decrease in the growth rate ($P < 0.0001$) and infectivity rate ($P = 0.001$ and $P < 0.0001$, respectively) for mutants I437M and I437L compared with the wild type and no significant difference in the case of the I437V and K436R mutants (Fig. 2c and d). Thus, the mutant viruses with lower replicative capacity (I437M and I437L) are those that were also less well recognized by CTL. These results indicate that the I437M and I437L B*13-RI9 escape mutants have reduced infectivity and viral replication compared with the I437V and K436R mutants, which maintained viral replication and infectivity profiles similar to those of the wild-type virus.

The I437M and I437L mutants have altered Gag protein profiles in viral particles and cell lysates. Next we investigated the mechanism underlying the fitness constraint of the Gag RI9 mutants. As a first step, we studied the relative levels of processed (p24) and unprocessed (p55) Gag polyprotein, since changes here would indicate either altered protease expression or altered polyprotein cleavage resulting from the HLA-B*13 escape mutations in p1. Jurkat cells were infected at an MOI of 0.001 with the different viral variants, and cell pellets and supernatant virus were harvested at days 1, 2, 3, 4, and 5. Proteins were extracted from cell lysates and virions and analyzed by Western blotting. Bands corresponding to p24 and p55 were visible only from day 4 of the time course (data not shown), indicating that only de novo synthesis of viral proteins is detected here. The data reveal a significant accumulation of unprocessed p55 Gag that is reflected in an increase in the p55/p24 ratio for the I437L and I437M mutant viruses at day 5 compared to the wild type in both infected cells (Fig. 3a and b) and viral particles (Fig. 3c and d). Meanwhile, the processing of the K436R and I437V mutant viruses was similar to that seen with the wild-type virus. Together, these data demonstrate that the low fitness escape I437M and I437L mutants have an accumulation of unprocessed p55 Gag protein.

I437M reduces ribosomal frameshifting efficiency. The RI9 variants map to a portion of the Gag-Pol ribosomal frameshifting signal of the HIV genome (Fig. 4). The slippery sequence and downstream stimulatory stem-loop structure are very conserved in HIV and allow precise control of the Gag/Gag-Pol ratio, the maintenance of which is crucial to viral infectivity, replication, and particle assembly (23, 24). Given the increased p55/p24 ratio for the I437L and I437M viruses detailed above and the replication defect, we hypothesized that these viruses may be compromised in ribosomal frameshifting.

In order to assess how these identified mutations might affect frameshifting efficiency, the frameshift region of the wild-type virus (2113 M-WT) and the mutants (I437L, I437M, K436R, and I437V) was cloned between the *Renilla* (R-luc) and firefly (F-luc) luciferase genes of dual-reporter plasmid p2luc (27) in such a way that expression of the R-luc–F-luc fusion protein (analogous to Gag-Pol) was dependent upon frameshifting at the inserted HIV signal. In vitro assays were carried out by translating mRNAs derived from EcoRI-digested p2luc/WT or a mutant derivative in RRL, and the non-

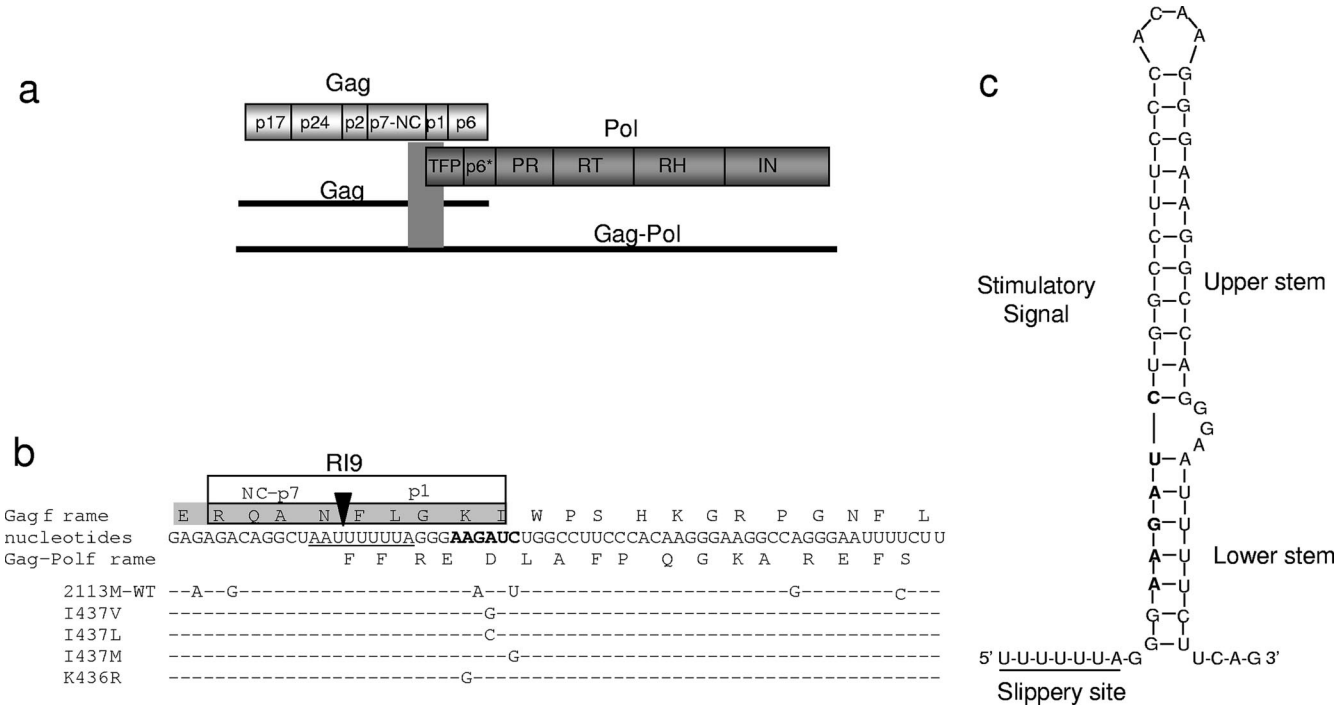

FIG. 4. Schematic representation of Gag and Gag-Pol polyproteins and locations of RI9 mutations. (a) Representation of HIV Gag and Gag-Pol polyprotein expression. The Gag protein consists of the following structural viral proteins, as represented in the top rectangle: p17 (matrix), p24 (capsid), NC-p7, p2 (spacer peptide), p1 (spacer peptide 1), and p6 (p6 gag). Pol expression is dependent on a −1 frameshift event at the overlap of the Gag and Pol reading frames. Gag-Pol contains TFP and p6* (p6 pol) and the viral enzymatic proteins PR (protease), RT (reverse transcriptase), RH (RNase H), and IN (integrase). Gray shading represents the protease cleavage site between NC-p7 and p1. (b) Alignment showing the Gag and Gag-Pol frame and nucleotide changes for the different mutants. The protease cleavage site NC-p7/p1 is indicated by a black arrow and gray shading, and the rectangle indicates the RI9 epitope. (c) Frameshift stem-loop structure model based on the NL43 sequence. The frameshift signal is composed of a uracil-rich slippery sequence (underlined) and a downstream stimulatory RNA stem-loop with upper and lower paired regions. The bold nucleotides are those implicated in B*13 RI9 escape.

frameshifted (39-kDa) and frameshifted (60-kDa) products were quantified by densitometry. In culture assays, the p2luc/WT plasmid or a mutant derivative was transfected into mammalian (Cos-7) cells and luciferase activities were measured at 24 h posttransfection. In order to quantify frameshifting efficiency in vivo, a "100% frameshift" control plasmid was prepared in which the R-luc and F-luc sequences were aligned in frame by the insertion of an A residue immediately after the slippery sequence. The in vitro assays are shown in Fig. 5a, and the frameshift efficiencies engendered in vitro and in culture are summarized in Fig. 5b. No substantial differences in frameshift efficiency, in RRL or Cos-7 cells, were observed for the wild type and the mutant derivatives, with the exception of I437M, where frameshifting was reduced to 58% of the wild-type value in Cos-7 cells and to 74% in RRL.

The extent to which these data are consistent with the predicted impact of the identified mutations on the thermodynamic stability ($\Delta G°$) of the frameshift stem-loop was then assessed with the *mfold* program (http://frontend.bioinfo.rpi.edu/applications/mfold/cgi-bin/rna-form1.cgi). The predicted thermodynamic stability was increased for the I437L (−26.10 kcal/mol) and K436R/I437L (−26.20 kcal/mol) variants and decreased in the I437M (−21.60 kcal/mol) and K436R/I437M (−21.70 kcal/mol) variants compared with wild-type 2113 M (−23.20 kcal/mol) (data not shown). In contrast, the I437V and K436R mutant viruses, the two most similar to the wild-type virus in replicative capacity, were predicted to show little difference in $\Delta G°$ compared with 2113 M-WT. The same results were obtained by modeling the stem-loop structures of the different variants (data not shown). Thus, the reduced frameshifting efficiency seen with the I437M variant is consistent with the predicted phenotype of this mutant. However, the I437L variant has a similar biological phenotype yet displayed no reduction in frameshifting efficiency. An alternative mechanism is likely to be involved that would explain the reduction in the viral fitness of the I437L variant that also, however, has the same ultimate effect as I437M of increasing unprocessed p55 Gag within infected cells and in viral particles.

DISCUSSION

These studies describe the impact of escape mutations on CTL recognition and on viral fitness driven by HLA-B*13-positive subjects within the Gag RI9 epitope. The amino acid substitutions selected within the epitope that affected CTL recognition the most, namely, I437M and I437L, were also those that reduced viral replicative capacity and infectivity in vitro. These mutations are located within the p1 Gag protein, which is involved in the viral ribosomal frameshifting control of the Gag/Gag-Pol ratio and which also contains a protease cleavage site at the NC-p7/p1 junction. Both mutations that reduced in vitro viral replicative capacity significantly resulted

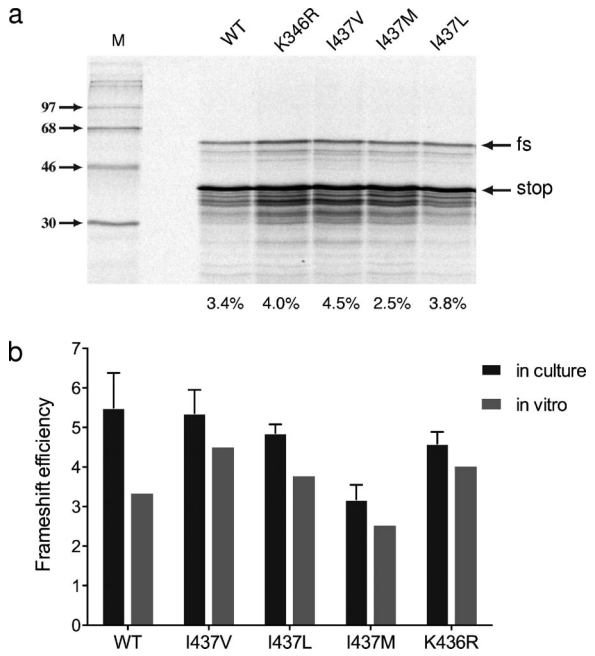

FIG. 5. Frameshift efficiency of RI9 escape virus. (a). In vitro frameshift assays in RRL. Plasmid p2luc/2113 M-WT or a mutant derivative was linearized with EcoRI and transcribed with T7 RNA polymerase, and transcripts were translated in RRL at a final RNA concentration of approximately 15 μg/ml. Products were labeled with [^{35}S]methionine, separated on a 10% SDS-polyacrylamide gel, and detected by autoradiography. The nonframeshifted (stop; predicted size, 39 kDa) and frameshifted (fs; predicted size, 60 kDa) species are indicated on the right. Lane M contains ^{14}C-labeled protein molecular size markers (Amersham Pharmacia Biotech). The percent frameshift efficiency measured for each mRNA is shown below each lane. The values on the left are molecular sizes in kilodaltons. (b) In-culture frameshift assays with Cos-7 cells. These data are presented graphically and include in vitro data for comparison. The mean frameshift efficiencies shown for Cos-7 cells were derived from in vivo experiments with six replicates for each plasmid. Error bars represent the standard error of the mean. WT, wild type.

in the accumulation of unprocessed p55 Gag in infected cells and in virus particles. However, only in the case of one of them, I437M, was evidence of reduced frameshifting efficiency observed. These data, therefore, further support the notion that immune control associated with specific CTL responses may, at least in part, be mediated by functional constraints operating against sequence change.

These data also illustrate the balance that exists between two opposing selection forces in HIV infection: on the one hand, those driving immune escape; on the other, purifying selection operating to conserve amino acid sequence and maintain viral fitness. The ideal result for the virus is complete escape at a negligible fitness cost, and recent studies have suggested that such a situation may apply more frequently in non-Gag epitopes (16, 32). As stated above, in the present study, the mutations in Gag that resulted in the most effective escape from CTL recognition were also those that had the greatest impact on viral replicative capacity.

Our studies show a preferential selection of escape variants with better replicative capacity despite only modestly reduced CTL recognition: virus containing the K346R or I437V mutation arises in 25% of clade C virus-infected B*13 individuals, while only 11% carry either I437M or I437L. Several factors are likely to contribute to the particular escape mutation selected in a given individual. These include the HIV-specific CTL responses operating in addition to the HLA-B*13-restricted RI9 response, as well as the extent of non-CTL anti-HIV immune activity, and virus-related factors such as viral sequence and viral load. The escape mutations having the greatest impact on viral fitness are likely to be outcompeted by escape mutations that have little impact unless sufficient selection pressure is imposed by the HLA-B*13-specific RI9-specific CTL in that particular subject. Furthermore, nucleotide bias during HIV replication may contribute to the preferential selection of I347V and K346R escape mutants. The greater tendency for G-to-A and A-to-G transitions during HIV-1 replication (34, 42, 44) might facilitate nucleotide changes of AA**A** to AA**G** for I347V and **A**AA to **G**AA for K436R, and with no fitness cost associated, these variants may be preferentially fixed in B*13 individuals as long as the potency of the RI9-specific CTL is reduced adequately.

To date, CTL escape mutations associated with a fitness cost demonstrated in vitro have been mapped to regions in the HIV p24 capsid only (9, 30, 45). Here, we identify CTL escape in a different region of the HIV proteome, in the small p1 Gag protein, that is also associated with a viral fitness cost. Recently, CTL escape driven by HLA-Cw*0102 in the p6 Gag protein has also been described, although the impact of this mutation on viral fitness was not determined (7). Although natural variants in this region of the HIV genome have been previously described (47), they have only been recently associated with CTL escape. Statistical analysis of Gag sequences in a large Vancouver cohort predicted escape and reversion within this same B*13-RI9 epitope (5).

The frameshift stem-loop located in p1 Gag is a secondary structure in the viral mRNA essential for the expression of Gag-Pol upon a −1 frameshift event (14) and appears to be very conserved in all of the HIV group M subtypes (1). Moreover, changes in the stability and structure of the frameshift stem-loop may alter ribosome pausing and disfavor frameshifting, thus reducing viral infectivity (2, 11). The frameshift stem-loop controls the ratio of Gag and Gag-Pol proteins expressed. Modification of the Gag/Gag-Pol ratio from the optimum of 20:1 has been found to reduce the level of virion production (46), and overproduction of Gag-Pol prevents correct virion formation and budding (24, 38). RI9 escape mutants map to the lower stem of the frameshift structure, and it has also been shown, by site-directed mutagenesis, that changes in the lower stem of the frameshift stem-loop can affect viral replication (11). Thus, it is possible that CTL mutations in this region of the virus may affect viral fitness.

To understand better the mechanism of the fitness cost to the I437M and I437L escape mutants, we therefore investigated whether the fitness cost to these RI9 mutants relates to alteration in protein maturation and to potential modifications in the frameshift stem-loop. Western blot quantification of p24 and p55, the unprocessed p24 precursor, showed an imbalance in the p55-to-p24 ratio for both the I437M and I437L low-fitness variants. Frameshift assays revealed that of the mutants, only the low-fitness I437M mutant had significantly reduced frameshift efficiency relative to the wild type. Thus, in the case

of I437M, these results support the notion that a reduction in frameshift efficiency may reduce Gag-Pol expression and the amount of active protease available for the cleavage of p55. In the case of the I437L virus, the mechanism associated with an in vitro fitness cost and the increase in p55 is unknown. However, there are a number of likely possibilities indicated by mutational analysis studies of the p1 region (21) which indicate effects on protein processing, RNA dimer stability, and virus infectivity. It is possible, therefore, that I437L may modulate the protease cleavage rate. Moreover, I437L is introducing changes not only in the *gag* but in the *pol* frame encoding the transframe protein (TFP). TFP is a highly conserve octapeptide important for the activation and regulation of protease activity (39), and it is possible that the changes in the TFP sequence introduced by I437L may affect the amount of active protease, reducing the cleavage activity and increasing the level of unprocessed Gag p55 protein (21).

In summary, this work suggests that the B*13-RI9 epitope may contribute to successful immune control in B*13-positive subjects because it is able to drive viral escape that can lead to an effect on viral fitness. The fitness cost of escape mutations, together with the breadth of Gag CTL responses available for HLA-B*13-positive individuals, will contribute to control of HIV replication. Furthermore, these studies provide the first evidence that mutations driven by escape from an epitope in a secondary structural region of HIV-1 can have an effect on viral fitness and highlight the importance of viral constraints in the selection of mutational pathways of CTL escape. These studies underline the tension that exists between the selection of escape mutations that reduce viral fitness and the need for the virus to evade potent CTL responses capable of effective viral suppression.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust (P.J.R.G.), Marie Curie program IEF contract 41811 (J.G.P), the Biotechnology and Biological Sciences Research Council (I.B.), and the HIVACAT project (J.M.P.).

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